Materials and Method

Note: To our best knowledge, this should represent an accurate description of the materials and methods required to reproduce our work. If any of the content on this page is difficult to interpret or should you have trouble repeating our work, do not hesitate to contact us as soon as possible in order for us to provide additional information and advice.

Entry Clone Source: Complex

Entry Clone Accession: n/a

SGC Construct ID: XX01BMPR1BA-c001

GenBank GI number: n/a

Vector: pFB-LIC-Bse. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

```
CCATGGGCCACCATCATCATCATCATTCTT
CTGGTGTAGATCTGGGTACCGAGAACCTGT
ACTTCCAATCCATGACTTACATTCCTCCTG
GAGAATCCCTGAGAGACTTAATTGAGCAGT
CTCAGAGCTCAGGAAGTGGATCAGGCCTCC
CTCTGCTGGTCCAAAGGACTATAGCTAAGC
AGATTCAGATGGTGAAACAGATTGGAAAAG
GTCGCTATGGGGAAGTTTGGATGGGAAAGT
GGCGTGGCGAAAAGGTAGCTGTGAAAGTGT
TCTTCACCACAGAGGAAGCCAGCTGGTTCA
GAGAGACAGAAATATATCAGACAGTGTTGA
TGAGGCATGAAAACATTTTGGGTTTCATTG
CTGCAGATATCAAAGGGACAGGGTCCTGGA
CCCAGTTGTACCTAATCACAGACTATCATG
AAAATGGTTCCCTTTATGATTATCTGAAGT
CCACCACCCTAGACGCTAAATCAATGCTGA
AGTTAGCCTACTCTTCTGTCAGTGGCTTAT
GTCATTTACACACAGAAATCTTTAGTACTC
AAGGCAAACCAGCAATTGCCCATCGAGATC
TGAAAAGTAAAAACATTCTGGTGAAGAAAA
ATGGAACTTGCTGTATTGCTGACCTGGGCC
TGGCTGTTAAATTTATTAGTGATACAAATG
AAGTTGACATACCACCTAACACTCGAGTTG
GCACCAAACGCTATATGCCTCCAGAAGTGT
TGGACGAGAGCTTGAACAGAAATCACTTCC
AGTCTTACATCATGGCTGACATGTATAGTT
TTGGCCTCATCCTTTGGGAGGTTGCTAGGA
GATGTGTATCAGGAGGTATAGTGGAAGAAT
ACCAGCTTCCTTATCATGACCTAGTGCCCA
GTGACCCCTCTTATGAGGACATGAGGGAGA
TTGTGTGCATCAAGAAGTTACGCCCCTCAT
TCCCAAACCGGTGGAGCAGTGATGAGTGTC
TAAGGCAGATGGGAAAACTCATGACAGAAT
GCTGGGCTCACAATCCTGCATCAAGGCTGA
CAGCCCTGCGGGTTAAGAAAACACTTGCCA
AAATGTCAGAGTCCCAGGACATTAAACTCT
GACAGTAAAGGTGGATACGGATCCGAATTC
GAGCTCCGTCGACAAGCTT
```

Final protein sequence (tag sequence in lowercase):

```
mghhhhhhssgvdlgtenlyfqsmTYIPPG
ESLRDLIEQSQSSGSGSGLPLLVQRTIAKQ
IQMVKQIGKGRYGEVWMGKWRGEKVAVKVF
FTTEEASWFRETEIYQTVLMRHENILGFIA
ADIKGTGSWTQLYLITDYHENGSLYDYLKS
TTLDAKSMLKLAYSSVSGLCHLHTEIFSTQ
GKPAIAHRDLKSKNILVKKNGTCCIADLGL
```

AVKFISDTNEVDIPPNTRVGTKRYMPPEVL
DESLNRNHFQSYIMADMYSFGLILWEVARR
CVSGGIVEEYQLPYHDLVPSDPSYEDMREI
VCIKKLRPSFPNRWSSDECLRQMGKLMTEC
WAHNPASRLTALRVKKTLAKMSESQDIKL

Tags and additions: mghhhhhhssgvdlgtenlyfq*sm. cleavable N-terminal hexahistidine tag.

Host: SF9 Spodoptera frugiperda Insect cells

Growth medium, induction protocol: Sf9 cells at a density of $2x10^6$/ml were infected with recombinant BMPR1B baculovirus (virus stock P2; 1ml of virus stock/100 ml of cell culture). Cells were shaken at 110 rpm at 27°C in an Innova shaker. After 48 hours post-infection the cultures were harvested by centrifugation for 20min at 6000rpm. Cell pellets from each 1L flask were resuspended in 15 ml binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole). Calbiochem protease inhibitor SET V was added to the cell suspension at a 1:2000 dilution. The suspensions were then transferred to 50 ml Falcon tubes, and stored at -20°C.

Extraction buffer, extraction method: The frozen cells were thawed and the volume increased to 100 ml with binding buffer. The cells were lysed using an Emulsiflex C5 homogeniser. The cell lysate was spun down by centrifugation at 21.5K rpm and 4°C for 1 h. The supernatant was recovered for purification.

Column 1: Anion-exchange for Nucleic acid removal with DEAE cellulose (DE52, Whatmann).10 g of resin was suspended in 50 ml 1 M NaCl, and then applied onto a 2.5 x 20 cm column. The resin was then equilibrated with 50 ml binding buffer prior to loading the sample.

Column 1 Buffers:
Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole, 0.1mM TCEP
Wash buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 25 mM imidazole, 0.1mM TCEP

Column 1 Procedure: The supernatant was first applied onto the column by gravity flow, which was followed by a wash with 50 ml wash buffer. The column flow-through and wash was directly applied onto a Ni-sepharose column.

Column 2: Ni-Affinity Chromatography. 6 ml of 50 % Ni-sepharose slurry was applied onto a 1.5 x 10 cm column. The column was equilibrated with binding buffer (25ml).

Column 2 Buffers:
Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole, 0.1mM TCEP
Wash buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 25 mM imidazole, 0.1mM TCEP
Elution buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 50 to 250 mM imidazole, 0.1mM TCEP

Column 2 Procedure: The flow-through from column 1 (DE52) was applied by gravity flow onto the Ni-sepharose column. The bound protein was eluted by applying a step gradient of imidazole - using 10 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 250 mM).

Enzymatic treatment: 0.1mg of TEV protease was added to the Ni-eluted protein to remove the tag.

Complex Assembly: BMPR1B was mixed with an excess of FKBP12 (see methods for FKBP12 below)

Column 3: Size Exclusion Chromatography - S75 HiLoad 16/60 Superdex run on ÄKTA-Express

Column 3 Buffers: Gel Filtration buffer: 300 mM NaCl, 50 mM Hepes pH 7.5, 0.5mM TCEP

Column 3 Procedure: Prior to applying the protein, the S75 16/60 column was washed and equilibrated with gel filtration buffer. The two proteins were mixed and concentrated to 3 ml using an Amicon Ultra-15 filter with a 10 kDa cut-off. The concentrated protein was directly applied onto the equilibrated S75 16/60 column, and run at a flow-rate of 1 ml/min. The protein was eluted at 58-68 ml. Fractions containing the protein were pooled together.

Mass spectrometry characterization: The purified protein was homogeneous and had an experimental mass of 38.267 and 12.038 kDa, as expected from primary sequences. Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation

and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% acetonitrile in water with 0.1% formic acid.

MATERIALS & METHODS FOR FKBP12 PRIOR TO COMPLEX FORMATION

Entry Clone Accession: BC005147

SGC Construct ID: FKBP1AA-c001

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:
ATGGGAGTGCAGGTGGAAACCATCTCCCCA
GGAGACGGGCGCACCTTCCCCAAGCGCGGC
CAGACCTGCGTGGTGCACTACACCGGGATG
CTTGAAGATGGAAAGAAATTTGATTCCTCC
CGGGACAGAAACAAGCCCTTTAAGTTTATG
CTAGGCAAGCAGGAGGTGATCCGAGGCTGG
GAAGAAGGGGTTGCCCAGATGAGTGTGGGT
CAGAGAGCCAAACTGACTATATCTCCAGAT
TATGCCTATGGTGCCACTGGGCACCCAGGC
ATCATCCCACCACATGCCACTCTCGTCTTC
GATGTGGAGCTTCTAAAACTGGAATGA

Final protein sequence (tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfqsmGVQVETI
SPGDGRTFPKRGQTCVVHYTGMLEDGKKFD
SSRDRNKPFKFMLGKQEVIRGWEEGVAQMS
VGQRAKLTISPDYAYGATGHPGIIPPHATL
VFDVELLKLE

Tags and additions: mhhhhhhssgvdlgtenlyfq*sm. cleavable N-terminal hexahistidine tag.

Host: BL21(DE3)-R3-pRARE2

Growth medium, induction protocol: A glycerol stock was used to inoculate a 25 ml starter culture containing LB media and 34 µg/ml chloramphenicol and 50 µg/ml kanamycin. The starter culture was grown overnight at 37°C with shaking at 250 rpm. A flask containing 1L LB media with 34 µg/ml chloramphenicol and 50 µg/ml kanamycin was inoculated with 5 ml of the starter culture. The 1L culture was incubated at 37°C with shaking at 160 rpm until an OD600nm ≥ 0.5 was reached. The flasks were then cooled down to 18°C and 0.5 mM IPTG added to induce protein expression overnight. Cells were harvested by centrifugation at 4500 rpm at 4°C for 15 min. The cell pellet was resuspended in 30 ml binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole), transferred to a 50 ml tube, and stored at -20°C.

Extraction buffer, extraction method: The frozen cells were thawed and 0.5 mM TCEP and 1 mM PMSF were added to the cell suspension. The cells were lysed by sonication over 12 min with the sonicator pulsing ON for 5 sec and OFF for 10 sec. The cell lysate was spun down by centrifugation at 21.5k rpm and 4°C for 1 h. The supernatant was recovered for purification.

Columns 1 and 2: FKBP12 was purified from the supernatant using the same column 1/column 2 protocol as shown above for BMPR1B. The two proteins were mixed as described above before further purification as described above.

Enzymatic treatment: 0.1mg of TEV protease was added to the Ni-eluted protein to remove the tag.

Crystallisation of the BMPR1B-FKBP12 complex: Protein was buffered in 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM DTT, 50mM L-arginine, 50 mM L-glutamate and concentrated to 15 mg/ml (calculated using an extinction co-efficient of 69330). 1 mM LDN-193189 was added to the final sample. Crystals were grown at 4°C in 150 nl sitting drops mixing 100 nl protein solution with 50 nl of a reservoir solution containing 20% PEG 3350, 0.20 M Na(malonate) pH 7.0. On mounting crystals were cryoprotected with mother liquor plus 20% ethylene glycol and flash frozen in liquid nitrogen.

Data Collection: Resolution: 2.05 Å resolution; **X-ray source:** Diamond Light Source, station I02, using monochromatic radiation at wavelength 0.979 Å